TECHNIQUE IN CONTRASTING MUCORS

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(WITH TWO FIGURES)

For the last two years a more or less intensive study of the sexual reactions between different races of mucors has been conducted. These reactions have been tested by growing the races side by side in culture dishes under suitable environmental conditions and observing the abundance of conjugations which take place between the races thus "contrasted." In making so large a number of contrasts, it has been necessary to develop a special technique in order to minimize the time consumed in the preparation and handling of the cultures, to obtain greater accuracy in making observations, and to avoid the various experimental errors to which one untrained in the work is liable. As has already been shown, the discordant results obtained by different investigators working with the same species of mucors may be explained by a difference in the technique employed. It has seemed desirable, therefore, to give in some detail the methods used before presenting the results of the investigations in a series of papers which it is hoped to publish shortly.

Gross cultures.—As a source of races to be investigated, gross cultures grown in the laboratory are more convenient than natural cultures found out of doors. Certain forms are characteristic of certain types of substrata. Most mycologists are familiar with the flora obtained from dung cultures made by placing bits of dung on filter paper above damp peat moss in a covered crystallizing dish. Brazil nuts have been found a constant source of certain forms such as *Cunninghamella bertholletiae*, *C. echinulata*, *Syncephalastrum*, and certain other species. In making gross cultures of these nuts it was found convenient to use chiefly the shells, since the meats furnish too luxuriant a supply of nutrient, so that *Rhizopus* is likely to overgrow the less vigorous forms in the culture. The nuts were cracked separately and their shells placed in piles

on filter paper above peat moss in shallow galvanized cake tins covered with glass plates. The shells of each nut were kept separate and placed near the edge of the culture dish in order to facilitate their examination with the hand lens. The hands and the nut-cracker were sterilized with alcohol after cracking each nut, in order to prevent an undue amount of infection of one nut from spores which may have been on the one cracked previously. It was the usual practice to reserve a culture dish for the nuts obtained from a single collection, despite the fact that many of these collections were from stores within a radius of thirty-five miles from New York City, and very probably had the same ultimate source.

Another prolific origin of mucors is soil obtained from different sources. It was most conveniently scattered on bread, which furnished a suitable medium for the germination and growth of the spores which the soil might happen to contain. The bread before using was sterilized in the autoclave without pressure for about 5 minutes, a longer time not being used since it might make the bread soggy. If the surface of the bread has become dry, it may be moistened slightly with sterilized water.

Stock cultures.—The races were obtained in pure cultures by making transfers from individual heads in the gross cultures. Since there is possibility of spores from another race being on the head from which the transfer was made, a second pure transfer was regularly made from an individual head in the first test tube, thus avoiding the likelihood of the stock cultures being mixtures of more than a single strain. As a precaution against too rapid drying out, the amount of agar flour in the stock cultures was raised from 2 to 3 per cent, and standard no. 230 nutrient, consisting of 2 per cent each of dextrose and dry malt extract plus 0.1 per cent meat peptone, was used.

Infection.—Knowledge of an investigator's methods may enable the impartial critic to judge whether the known sources of experimental error are properly guarded against. To the bacteriologist and the student of fungi in pure cultures, one of the greatest sources of error is infection. When this infection is caused by a form different in appearance from the species under cultivation, the presence of the foreign growth is readily recognized. Infection

is less readily recognized, however, when the invading growth is another race of the same species. *Syncephalastrum* and *Cunninghamella* are forms which bear their spores externally and shake them off at the slightest touch or breath of air. When a dish containing a mature culture of these species is opened, the spores may be seen to rise in clouds. The spores of *Rhizopus*, although inclosed in a sporangium, are readily scattered into the air by the rupture of the brittle sporanguim wall, and in consequence *Rhizopus* also is a common source of infection. That the air may be filled with viable spores of both sexes of *Cunninghamella* has been shown by exposing Petri dish cultures for a short time and growing them at a temperature suitable for zygospore formation. In the laboratory where *Cunninghamella* had been recently grown, it was always possible to find growths of the same species in Petri dishes exposed in this way, and frequently both the sexual races were obtained, as shown by the production of their zygospores. Experience has shown, therefore, that in working with *Cunninghamella* one must observe greater precautions to avoid air-borne infection with other strains of the same species than is necessary when working with many other forms.

Not only are cultures of *Cunninghamella* especially liable to infection with spores of the same species, but the spores of this species which gain access to a mature culture are able to germinate and grow on the aerial mycelium which they infect. With most mucor species, vigorous growth on the nutrient substratum is necessary before zygospore formation is possible. With *Cunninghamella*, however, connection with the nutrient agar does not appear to be necessary in order to allow the mycelia to assist in zygospore formation, since if spores of one sex are planted on an aerial growth of the other sex, zygospores are likely to be produced. This peculiarity of *Cunninghamella* was responsible in the earlier cultures for the appearance of zygospores where they should not be found on the theory of sexual dimorphism, and led to a repetition of the first series of cultures and a revision of the technique.

NUTRIENT MEDIA.—The method of growing and testing races of *Cunninghamella* for sexual reactions was the same as that adopted for other forms, with such slight modifications as the peculiarities of

the individual species demanded. Two per cent agar with the addition of different nutrients was used as a substratum in all cases. The formula varied with the different species tested. No. 230 standard stock nutrient (already described) was used in the tests when possible. Before starting an extensive series of tests of a given species, however, the effects of a number of different nutrients were tested, and the one chosen which was able to support a relatively abundant production of zygospores. For "imperfect hybridization" reactions no better nutrient was found than no. 362, which is a milk whey agar consisting of 2 per cent agar, 1 per cent dextrose, and 2 per cent dry milk whey powder. Some species form zygospores at laboratory temperatures, while others require a higher temperature for sexual reproduction. The species of *Cunninghamella* investigated belong to the latter class, and accordingly tests of this genus have been grown in the incubating oven at temperatures between 24° C. and 28° C.

CULTURE DISHES.—A suitable culture dish is a matter of some importance, especially when large numbers of cultures are handled together. It should be relatively small in order to economize space, and yet should provide sufficient surface of the nutrient medium to support vigorous growth of the two contrasted mycelia. The danger of infection must be reduced to a minimum, and yet the dishes should be such as to be manipulated easily in being filled with nutrient, inoculated with the races to be tested, stored during incubation, and examined under the microscope. Test tubes, although fitted for holding material in stock cultures, require considerable time to be plugged, filled, and sterilized, and moreover cannot easily be handled for observation under the microscope. Petri dishes, while in many ways superior to test tubes, are expensive, do not stack well, and are furnished with a loosely fitting rimmed cover. The Syracuse watch glass with ground rim for pencil labeling has been found to satisfy all the requirements of a safe and convenient culture dish, and has been used almost exclusively in recent years in testing contrasts between different races. They are conveniently handled in stacks of five, four dishes for cultures and an empty one for the top cover. Each dish, except the bottom one, serves thus as a cover for the one

below. A single stack, therefore, can be used in testing each of two races by contrasts with a plus and a minus tester. The stacks of culture dishes are dry sterilized, and when cool filled with sterilized nutrient agar slightly above the melting point. If the agar is too warm, moisture may condense on the covers and later drop on to the surface of the hardened agar. The process of pouring agar into the dishes is carried on in a special culture room to minimize danger of contamination. The cultures are ready for inoculation as soon as the agar has begun to harden. Pouring agar into sterile dishes rather than sterilizing the dishes after they have been filled not only saves considerable time in the process, but avoids the spattering of nutrient on the edges of the dishes, which is likely to ensue when they are autoclaved, and is a ready source of infection. It has not been found necessary to use cleared agar, but the sediment at the bottom of the flask from which the stacks are poured has usually been discarded. This sediment may be conveniently anchored to the bottom by cooling the flask when full in a shallow pan of water.

INOCULATIONS.—Since forty or more individual races in test tubes may be used in inoculating a given series, it is obvious that some precautions are necessary to avoid contamination from spores falling from these tubes, or from the weft of spore material taken from them for inoculation. Exposed Petri cultures have shown that this danger is great if not guarded against. In making inoculations, great care was taken never to expose to the air in the inoculating chamber any spore material in a dry condition. A fairly large piece of rather soft agar was transferred with a flamed platinum needle or spatula to the tube from which it was wished to obtain a transfer. The piece of agar was cautiously pushed against the spore material, and thoroughly mixed with it, care being observed that no dry filaments adhered to the needle or to the mixture of agar and spore material to be used as the inoculum. Moreover, the cotton plug was not removed suddenly from the test tube, since if this is done spores are likely to be shaken into the air, sometimes in visible clouds. After the inoculation the needle was not at once flamed, as the heated inoculum may sputter and scatter bits containing viable spores. The needle, therefore, was left in a tube of about 80 per cent alcohol while the label was

being written on the culture. This alcohol bath helps to sterilize not only the needle but also the base of the needle holder, which cannot readily be flamed, but which may carry spores from the test tube cultures. The alcohol was burned off and the needle flamed before a new inoculation was made. A layer of shot will be found convenient to weight the jar of alcohol and to receive the point of the needle. It is a rule of the laboratory never to lay a test tube down from which a transfer is being made until the label is written on the new culture.

The first race to be tested was inoculated in a streak at the left and at the right respectively of the first and second culture dishes in the stack. Similarly the second race was streaked at the left of the third dish and at the right of the fourth dish. In like manner other stacks were inoculated with the remaining races to be tested, so that finally a series of stacks was secured with two of the dishes streaked with one race and two dishes with another race. They were then ready to be planted with the testers, which are most conveniently a pair of races of opposite sex. In such a case, the plus was streaked on the right of the first and third culture dishes of each stack, and the minus on the left of the second and fourth dishes. Each dish, therefore, contained a tester and a race to be tested, and each stack accordingly completely tested two races. An advantage in choosing a plus and a minus race as the two testers in a series was that they could be planted together as controls for the production of zygospores. They may be grown in duplicate, and a pair of dishes with nutrient but without inoculations may complete the control stack and give evidence of the amount of infection to which the cultures are liable. In inoculating the testers a larger amount of spore material was rolled up on the needle, which sufficed without renewal for the inoculation of 30 or 40 dishes. With practice the process of inoculation could be carried on with relative rapidity. In inoculating a culture with more than a single race, the needle should be kept on its own side, to decrease the likelihood of spores falling upon a part of the substratum reserved for another race.

When "imperfect hybridization" was expected, the tester and the race to be tested were streaked so that the lines of inoculation formed a V instead of running parallel. In consequence, the two

mycelia met sooner in their growth at one end of the line of contact than at the other, and thus offered a greater area at a given time for the observation of sexual reactions which may soon be covered by the later growth of the mycelium. Obviously the angle of the V must be left open, and care taken in planting a tester to avoid touching with the needle the previous line of inoculation. The distance between the points or lines of inoculations may be a matter of some importance, since certain forms fail to produce zygospores in the line near the inoculations, while others produce them only within a distance of a few millimeters from the inoculations.

After inoculation, the stacks are stored in the incubating oven if greater than laboratory temperature is necessary. If the stacks are inverted until the mycelium covers the agar, the danger is avoided of water condensing on the dishes above and falling upon the cultures, with a consequent running together of the recently made inoculations. It may be found desirable to have jars of water on the culture shelves to prevent the nutrient from drying out before they are finally recorded. Before the cultures had matured, they were inspected to see whether any inoculation had failed to grow. With *Cunninghamella* the inspection was more thorough than with most other forms, and consisted in an examination for beginnings of conjugation such as are seen in "imperfect hybridization." Apart from this early inspection on the first or second day after inoculation, and before there is much danger in disseminating spores from the cultures into the air, the stacks were not opened before being sterilized in the autoclave, generally on the seventh or eighth day. The heat of the autoclave melts the agar and tends to glue the filaments to the bottom of the dish above. It was found convenient, therefore, to have the stacks inverted during sterilization, with a pan below to catch the melted agar. Just before cooling the stacks were erected and each dish lifted in succession. Any of the cultures still adhering to their covers after this procedure may be freed by the use of alcohol. Keeping the dishes closed while the spores are likely to be shed reduces the amount of infection of the laboratory air, which is probably impossible to prevent entirely when a species like *Cunninghamella* or *Syncephalastrum* is cultivated in a wholesale manner. Fig. 1 shows a series

of empty stacks in the inoculating room ready for pouring. A tube of alcohol is seen at the right holding the inoculating needles.

EXAMINATION OF CULTURES.—The cultures were examined under the binocular microscope by the transmitted light of a substage electric lamp. In some species the mycelium and spores above the substratum hid any zygospores which might be present, and necessitated manipulating the culture before examination. Wetting with alcohol, pressing down the aerial growth with the finger, and even washing the spores away under a jet of water occasionally

FIG. 1.—Inoculating chamber with stacks of culture dishes ready for pouring

was found necessary. The relative abundance of zygospores when present is shown by the grades *A* to *D*. *A* indicates about the maximum number of zygospores to be expected of the species under the given environmental conditions, while *D* indicates generally less than a dozen zygospores in the whole culture. Absence of zygospores is indicated by *O*. Naturally the zygospores would be expected to form in greatest numbers at the line of contact between the opposing mycelia, and in some species produce a sharply defined dark line. In forms like *Rhizopus* and *Cunninghamella*, in which the zygospores are produced on branching aerial filaments, the zygospores may spread from this median line until ultimately

they are scattered through the whole culture. They should not be least abundant in the center, however, and a very few isolated zygospores at some distance from the line of contact make one suspicious of possible infection and demand a repetition of the culture.

In the study of incompleted sexual reactions such as are found in "imperfect hybridization," greater care must be exercised than in the observation of zygospores, since the cultures have not been sterilized, and infection may take place in an early examination of the dishes and be the cause of the sexual reactions seen when the cultures are looked at later. At first glass plates were used to cover the cultures, to prevent the delicate filaments from drying and collapsing while they were being examined. This entailed cleaning and sterilizing the plates in alcohol and afterwards drying them, and consumed more time than the procedure finally adopted. By this method a stack was placed on a sheet of wet blotting paper and covered with a crystallizing dish lined with moist paper, thus forming a humidor for the cultures. The stage of the binocular microscope was covered by several layers of wet blotting paper perforated to match the opening in the stage for the entrance of light. A collar of moistened blotting paper formed a moist chamber with the wet paper on the stage and allowed the examination of a culture to continue for some time without collapse of the hyphae. The cover was removed from the stack in the humidor formed by the crystallizing dish and placed upside down on the table. The bottom of the first culture dish was swabbed with a pledget of cotton soaked with alcohol before being put in the moist chamber formed on the stage of the binocular, and after being examined was placed upside down on the inverted cover. The second, third, and fourth cultures were treated in a similar manner, except that the bottom of the fourth culture, which had not been in contact with any culture below it, was not treated with alcohol. After the last dish had been removed from the humidor and examined, it formed the last member of an inverted stack, and a new stack was placed in the humidor ready for examination. From time to time it was found necessary to re-wet or renew the blotting paper on the binocular. The paper on the stage is theoretically capable

of transmitting spores to the cultures examined, but when the dishes were treated with alcohol in the manner indicated, it has not proved a source of error. Fig. 2 shows the arrangement of apparatus in an examination of cultures. At the left are two trays with stacks of cultures ready for examination. Next toward the right is a glass plate containing the humidor and the inverted members of a stack already examined. On the stage of the binocular is a culture under examination. The fifth dish of the stack not yet examined is covered by the humidor. At the extreme right is a series of inverted stacks which have already been examined. The

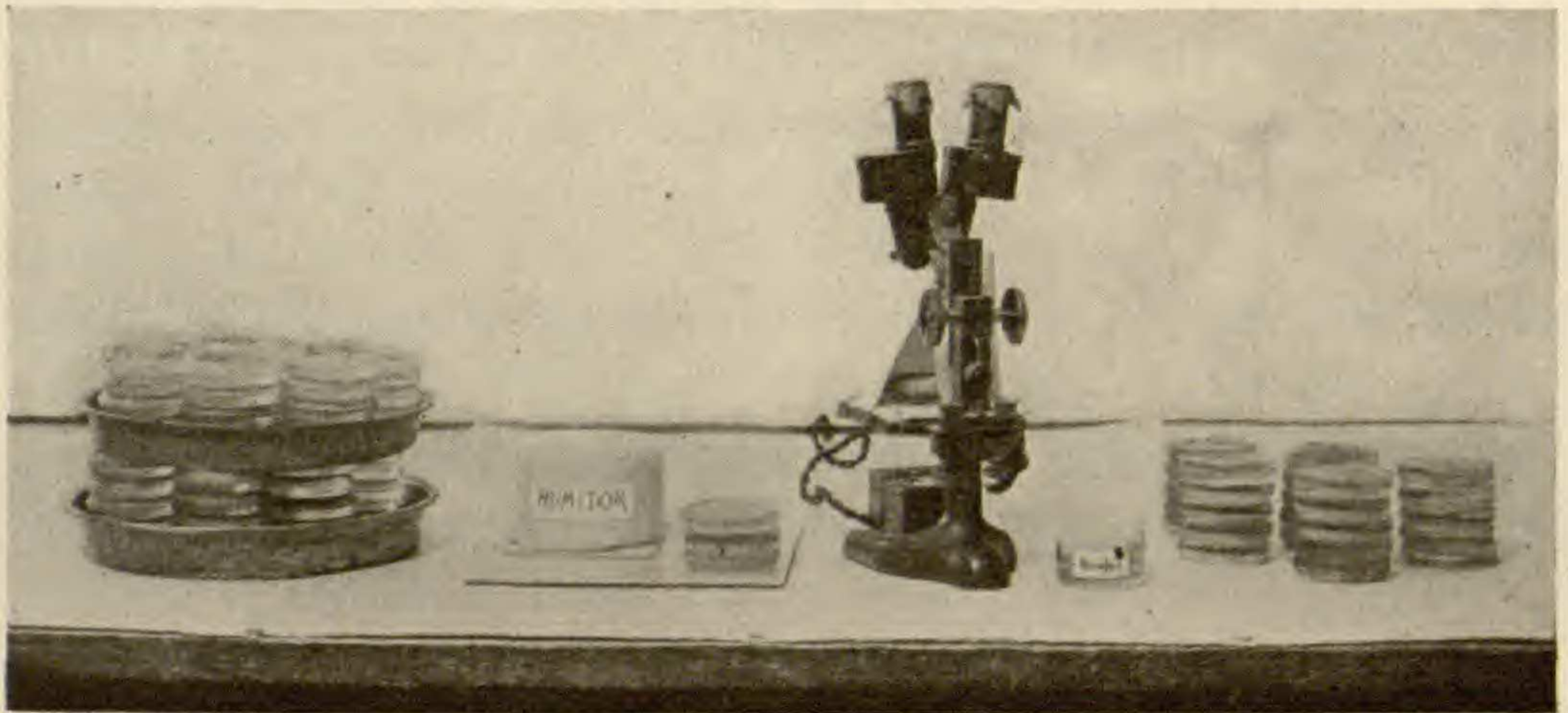

FIG. 2.—Culture dishes and apparatus used

stender dish marked "alcohol" contains a wad of cotton for swabbing the dishes. Blotters are seen on the stage of the binocular as well as the paper collar which partially incloses the culture under examination.

In earlier work on "imperfect hybridization" the bits of mycelium at the line of contact between two races were removed with needles and teased out for examination under the compound microscope in order to determine the presence or absence of sexual reactions. Unless stages in conjugation are relatively abundant, they are much more likely to be missed by this method than when examined at the proper time in the living condition under the binocular. The time of examination, however, must be well chosen, since if an examination is attempted after the line of contact between the two

races has become grown over, even a relatively strong reaction may not be evident. The growth of only a few hours may render a regrading of a culture impossible.

In the foregoing pages some of the sources of experimental error likely to be encountered in studying the sexual reactions between races of mucors have been pointed out, and the technique which a familiarity with these sources has led the writers to adopt has been outlined. What has been written may serve as an introduction to a paper to follow in this journal, in which it may be possible to show that the discordant conclusions reached by certain investigators working with the same material may have been due to differences in the methods employed.

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